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TITLE: Evaluation of Human Adipose Tissue Stromal Heterogeneity in Metabolic Disease Using Single Cell RNA-Seq

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14. ABSTRACT

We have developed a robust protocol to generate single cell transcriptional profiles from subcutaneous adipose tissue samples of human subjects using Drop-seq, a newly developed, cost-efficient method of highly parallel genome-wide expression profiling using nanoliter droplets. We have collected subcutaneous adipose tissue samples from multiple individuals and generated single-cell profiles for ~6000 cells. Preliminary analysis demonstrates expression profiles can be used to cluster individual cells into distinct cell types in an unbiased fashioned. We recover expected cell types known to be contained within adipose tissue and suggest new cell types and subtypes that had not previously been described. We can confirm many previously defined transcriptional markers for known cell types and discover many specific novel ones as well. These data provide a comprehensive transcriptional atlas of subcutaneous adipose tissue cell types that will provide molecular handles to understanding and manipulating each cell types function. These results are hypothesis-generating and provide the foundation for future studies that will 1) validate the role for newly identified mediators of obesity and insulin resistance in animal models and 2) examine novel targets against which we can design therapies to combat obesity and its related complications.

15. SUBJECT TERMS

Obesity, Type 2 Diabetes Mellitus, Insulin resistance, Adipose, Stromal vascular fraction

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INTRODUCTION:

The overall goal of this proposal is to determine how individual cell types within human adipose tissue interact to regulate adipose tissue physiology. Specifically, we will develop the molecular and analytical tools to identify and classify the identity and function of individual cell types within the adipose tissue stromal vascular fraction (SVF) in an unbiased fashion using single-cell transcriptional profiling. By comparing adipose tissue samples from a range of healthy and diseased individuals, we aim to determine how individual cell types work in concert to maintain adipose tissue health, and how this cellular network is compromised with chronic overnutrition (obesity).

KEYWORDS:
Obesity, Diabetes, Insulin Resistance, Adipose, Adipocytes, Stromal Vascular Fraction, Single-cell RNA-seq, Transcriptional profiling, Drop-seq

ACCOMPLISHMENTS:

What were the major goals of the project?

Goal	Milestone/ Target Date	Completion Date	% completion
Specific Aim 1: Comparing SVF from lean and obese individuals	Months		
Milestone Achieved: HRPO/ACURO Approval	3	01/14/2016	100%
FACS initial SVF samples (6 human subjects, 6000 cells) into individual plates	3-6	04/2016	100%
Generate Single-cell RNA-seq Libraries and Sequence	3-6	05/10/16	100%
Analyze initial 6000 cells for overall cellular complexity and evaluate pilot to determine how many cells will be needed per sample (Monocle, Cufflinks software)	6-9	06/2016	100%
Milestone Achieved: determination of number of cells per sample required	*9*		75%
FACS further obese/lean SVF samples (5-20 human subjects, see proposal for discussion of targeted number)	9-12		50%
Generate Single-cell RNA-seq Libraries and Sequence from further samples (directly above)	12-18		50%
Milestone Achieved: Single-cell RNA-seq of SVF from target number of lean/obese individuals (see proposal for discussion of targeted number)	12		50%
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur in lean and obese groups and determine gene and cellular networks governing phenotypes (Monocle, Cufflinks, GSEA, ARACNE software)	9-18		25%
Aim1b: Comparing SVF from insulin sensitive and insulin resistant subjects			
FACS further insulin sensitive and insulin resistant SVF samples with generation of Single-cell RNA-seq Libraries and sequence	9-12		25%
Milestone Achieved: Single-cell RNA-seq of SVF from target number of insulin sensitive and resistant individuals (see proposal for discussion of targeted number)	12		25%
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur in insulin sensitive versus insulin resistant groups and determine gene and cellular networks governing phenotypes, as above	9-18		25%
Specific Aim 2: Use single-cell Single-cell RNA-seq to profile SVF from paired subcutaneous and visceral fat samples			
Compare paired human SVF samples from			

subcutaneous and visceral depots			
FACS paired samples (12 human subjects) into individual plates	6-9		0%
Generate Single-cell RNA-seq Libraries and Sequence	9-12		0%
Milestone(s) Achieved: Single-cell RNA-seq of subcutaneous versus visceral SVF from 12 (see proposal for discussion of targeted number)	12		0%
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur between subcutanesous and visceral depots and determine gene and cellular networks governing phenotypes	9-18		0%

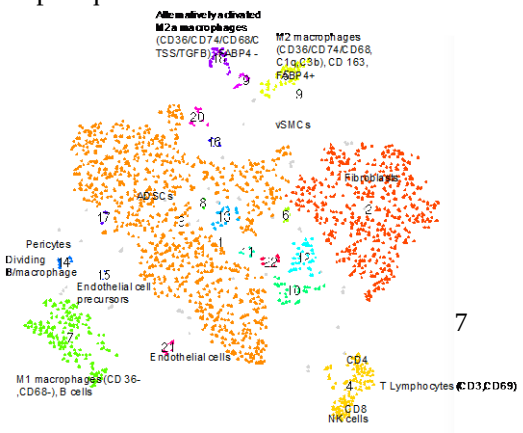
What was accomplished under these goals?

During initial phase of this grant, we have focused on technology and protocol development for preparing the stromal vascular fraction from human adipose tissue samples in a way that is amenable to generate single cell transcriptional profiles. We had difficulty adapting the SCRB-Seq protocol which we initially described in our grant application to human adipose tissue for a number of reasons. First, SCRB-seq had been successfully used on *in vitro* derived preadipocyte cells, which grow in monolayer and are easily dissociated (generally with limited <5 minutes of trypsin digestion). Dissociating individual SVF cells from whole tissues efficiently to ensure RNA quality requires a much longer enzymatic digestion time with negative consequence on RNA quality.

During the time we were struggling with a cell preparation method for SCRB-seq, we were able to start a collaboration with Evan Macosko and Steve McCarroll across the street, who had developed Drop-seq, a novel method to profile genome-wide expression in individual cells in highly parallel, cost-efficient manner using nanoliter-sized droplets (Macosko et al, 2015, *Cell* 161, 1202–1214). The Drop-seq system provided a number of benefits over SCRB-seq. First, it had been used to assess a more heterogeneous *in vivo* tissue (mouse retina). Second, the cost per cell estimates are an order of magnitude less than that of SCRB-seq after initial investment. Third, it obviates reserving excess FACS facility time(there is a huge variation in when human samples become available and how long they take to process, so to ensure FACS machine would be available, we often had to reserve 8 hours, even if only using machine for 2 of those hours). The decreased per cell cost would provide with ability to both more exhaustively assess SVF content and/or perform more individual samples.

A significant activity of this reporting period has been to set up the Drop-seq methodology in our laboratory and adapt it to human adipose SVF. We first performed trial experiments on the mouse arcuate nucleus of the hypothalamus (an area known to be important in appetite and weight regulation) to troubleshoot the method, given ease of access to that tissue. Using these pilot experiments, we have successfully set up all aspects of Drop-seq method, including microfluidic separation of individual cells into nanoliter droplets, parallel generation of individual cell RNA-seq libraries, and sequencing and analysis pipelines to be able to analyze such complex data.

The next major activity of this reporting period has been to adapt Drop-seq to human adipose tissue SVF, which has included a number of challenges. First, the scale of tissue preparation (sometimes kilograms of human adipose tissue compared to grams of mouse hypothalamic) has required protocol development to make sample preparation more efficient and scalable. SVF preparations from blood also contain a high number of



erythrocytes (>90% of indivudal cells), which we are not interested in assessing as they are not thought to be transcriptionally dynamic. We have had to test a number of erythrocyte removal techniques including selective centrifugation, lysis, and antibody-mediated removal to determine the right combination that preserves RNA quality in remaining cells (erythrocyte lysis releases factors that inhibit subsequent cDNA synthesis in remaining cells). The protocol has improved to the point that we have now successfully prepared high quality single cell profiles from a number of individuals. We show unbiased clustering of the first 4600 cells as well as provisional cell type assignments based on their transcriptional markers. We are able to recover, even in this limited number of cells most expected cell types (pre-adipocytes, macrophages, B cells, T cells, endothelial cells, vascular smooth muscle cells and pericytes, as well as subtypes of some of these cell types (eg. M1 and M2 macrophages). We believe with increasing numbers of cells from increasing numbers of individuals, we will be able to further subtype many of these cell types as well as find rare cell types that do not separately cluster without enough representation).

What opportunities for training and professional development has the project provided?
I take part in a working group based on this project including a Boston single-cell working group.

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
We plan to increase adipose samples in next reporting period. We will collect samples from an additional 5 lean, 5 obese, 5 insulin sensitive, and 5 insulin resistant individuals. Pooling cells from all individuals, we will perform iterative unbiased clustering analyses to determine transcriptionally distinct cell types. Using this atlas, we will define markers for individual cell types and determine whether any cell types correlate with any of the phenotypic parameters we collect (BMI, HOMA-IR, serum cholesterol triglyceride, CRP). We will also compare gene expression from individual cell types from lean vs. obese and insulin sensitive vs. insulin resistant subjects to determine how each individual cell type within adipose SVF responds transcriptionally to their metabolic phenotype.

Lastly, we will collect paired omental and subcutaneous adipose samples from subjects having abdominal surgeries to determine the minimum tissue input required for Drop-seq assessment of individual SVF cells (these samples will be 5-10g rather than the 200-2000g subcutaneous fat samples we receive from cosmetic surgeries). These experiments will provide assessment of feasibility of using Drop-seq to characterize visceral SVF and to assess differences between subcutaneous and visceral depots.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
We have made critical strides in developing a protocol to be able to assess individual cell type transcriptional profiles in adipose tissue SVF from human samples during this reporting period. By producing an atlas of cell types within human adipose SVF, we provide a delineation of the "parts" that make up adipose tissue as well as what are important genes that identify them. This should provide the field with molecular handles to better understand the and manipulate the function of specific cell types within this niche. Further, cell-type specific profiles can be used to better interpret specific cell types that may mediate genome wide association signals. By comparing the relative profiles of these cell types across individuals, we can also determine specific genes in specific cell types that correlate with metabolic characteristics such as obesity, diabetes, and cholesterol levels to better understand how specific genes and cell types modulate these metabolic characteristics.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change:
As above, we have switched our technique for single cell RNA-seq profiling from SCRB-seq to Drop-seq due to improvements in efficiency, cost, and feasibility.

Delays:
We encountered a delay in production phase due to our preliminary SCRB-seq trials not working and the set-up time involved in switching to Drop-seq. We have also encountered some recent delays in expected patient accrual of adipose tissue samples, as over the summer fewer cosmetic surgeries occur at our institution. We anticipate an increase in number of samples in the remaining months to collect the samples proposed in initial proposal.

Changes that had a significant impact on expenditures:
The change from SCRB-seq and Drop-seq techniques required moving funding from initial proposal of outsourcing library construction and sequencing costs to the Broad Institute to performing Drop-seq library construction and sequencing within our lab. This meant removing FACS core funding, purchasing of a new thermocycler in order to perform the library construction, and moving some of initial SCRB-seq costs to supporting the research assistant who is generating the libraries and sequencing them. As Drop-seq is more cost-efficient on a per cell basis, we anticipate we will be able to increase the number of SVF cells we are assessing per subject to 5,000-10,000 cells for at least 10 individuals (our initial estimate with SCRB-seq was to profile 21,000 cells total).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects:
none, IRB approval date: 1/14/2015

Significant changes in use or care of vertebrate animals:
N/A

Significant changes in use of biohazards and/or select agents:
N/A

PRODUCTS:

Publications, conference papers, and presentations

Journal publications: Nothing to Report. (Manuscript based on pilot arcuate hypothalamus data is under review at *Nature Neuroscience*)

Books or other non-periodical, one-time publications: Nothing to Report

Other publications, conference papers, and presentations: "A Transcriptomic Atlas of Arcuate Cell Types", Boston Single Cell Working Group, (Local meeting)

Website(s) or other Internet site(s):
Nothing to Report

Technologies or techniques:
Nothing to Report

Inventions, patent applications, and/or licenses:
Nothing to Report

Other Products:
Database of human SVF cell types, mouse arcuate cell types. Upon publication, these transcriptional data will be provided to the public via NIH's GEO database.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Linus Tsai
Project Role:	PI
Researcher Identifier	ORCID ID 0000-0002-0134-6949
Nearest person month worked:	3.6
Contribution to Project:	Performing patient recruitment, sample collection and processing, Dropseq, and directing library construction, sequencing and analysis
Funding Support:	Boston Area Diabetes Endocrinology Research Center Pilot and Feasibility Grant (NIH 2P30DK057521-16), Boston Nutrition and Obesity Research Center Core Grant (5 R01 DK 087092-05)

Name:	Anna Lyubetskaya
Project Role:	Computational Biologist
Researcher Identifier	eracommons: alyubets
Nearest person month worked:	1.2
Contribution to Project:	Ms. Lyubetskaya has performed computational analysis pipeline and developed methods for analyzing single cell transcriptional data
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Yes, for Linus Tsai, two new grants have been funded.
4/1/2016-3/31/2017 A cellular and molecular atlas of the arcuate hypothalamus using Dropseq
NIH 2P30DK057521-16 (Habener)
Consortium PI ($30,000)
This pilot and feasibility grant award supports development of Drop-seq capability to create a gene expression and cell type atlas for the arcuate nucleus atlas of cell types within the arcuate-median eminence complex using single cell transcriptional profiling.

4/1/2016-3/31/2017 Functional Genomics and Bioinformatics Core
PI Rosen 5 R01 DK 087092-05 (Rosen)
5P30DK046200 (Fried)
Role: Assistant Core Director ($81,920 DC/$58,036 IDC)
The goals of this Core are to provide access to state-of-the-art methods to members of the BNORC community, in order to enhance and facilitate a deeper understanding of how metabolic disease develops, and how it can be diagnosed and treated.

What other organizations were involved as partners?

Organization Name: Broad Institute
Location of Organization: Cambridge, MA
Partner's contribution to the project: Facilities (Provide computing infrastructure for analyses)

SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS: N/A
QUAD CHARTS: N/A

APPENDICES:
Nothing to report